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Analysis of the oligosaccharides in Japanese rice wine, sake, by hydrophilic interaction liquid chromatography–time-of-flight/mass spectrometry

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A traditional Japanese alcoholic beverage, sake, contains several oligosaccharides, which are associated with the taste of sake; however, little is known about the specific molecular species and concentrations of oligosaccharides in sake. Here, we developed an analytical method using hydrophilic interaction liquid chromatography–time-of-flight/mass spectrometry (HILIC-TOF/MS) which successfully detects the oligosaccharides in sake. A series of oligosaccharides with successive degree of polymerization (DP) values up to 18 were identified in sake for the first time, which we have named sake oligosaccharides (SAOs). The concentrations of the SAOs with DP = 3–8 were estimated to be in the range of 200–2000 ppm. Quantitative analysis of 6 different sake samples for SAOs with DP = 2–8 and the other saccharides showed that the amount of each SAO differs significantly among the sake samples. Enzymatic digestion analysis suggested that the SAOs are probably branched maltooligosaccharides in structure, which are resistant to β-amylase.

[**Key words:** Sake; Oligosaccharides; Rice; Branched-saccharides; Hydrophilic interaction liquid chromatography–time-of-flight/mass spectrometry]

The traditional Japanese rice wine, sake, is made by a characteristic fermentation process known as a multiple parallel fermentation; saccharification of the rice starch and alcohol fermentation occur at the same time in a sake mash. The rice starch is digested into oligosaccharides and glucose by the concerted action of the fungal enzymes, α-amylase, glucoamylase, and α-glucosidase. Glucose is subsequently catabolized by yeast into ethanol. As both saccharification and alcohol fermentation proceeds in parallel, appropriate control of both reactions is necessary to make sake with a balanced taste. The main index characterizing the type of sake is the sake meter value that indicates the degree of dryness or sweetness of sake. Sake meter value is the specific gravity determined mainly by the amount of alcohol and saccharides in the sake. Therefore, analysis of the saccharide content in sake mash or sake is of significant importance for controlling the fermentation and taste of sake.

Most of the non-volatile components in sake are saccharides with concentrations in the range of 3.5–7.5% (w/v) (1). Among the saccharides in sake, glucose is generally the most abundant with an estimated concentration in the range of 1–4% depending on the type of sake (2). Disaccharides such as isomaltose, sakebiose (nigerose), and kojibiose have been reported to be present in sake with the total amount reaching 2000–8000 ppm (3–5). Those saccharides are thought to be generated by the transglycosylation activity of α-glucosidase produced by the *koji* mould, *Aspergillus oryzae* (6,7). For the oligosaccharides with degree of polymerization (DP) over 2, isomaltotriose, panose, isomaltotetraose (DP 3), and isomaltopentaose (DP 4) were shown to be present in sake (5,8,9). The total amount of the oligosaccharides with DP over 3 in sake is estimated to be 2000–5300 ppm (8,10), which is more than the ~1500 ppm of amino acids (11). In spite of the importance of oligosaccharides in sake brewing and quality, the molecular species, the composition, and the concentration of oligosaccharides in sake remain unknown except for those with DP less than 4. Many of the important studies about oligosaccharides in sake were reported decades ago; oligosaccharides with DP over 3 were not precisely detected due to the limitations on resolution and sensitivity for the paper chromatography analysis. Thus, we focused on characterizing the oligosaccharides in sake using recent analytical technology in this study.

In previous studies, several different analytical methods have been used to separate and measure oligosaccharides in sake and other alcoholic beverages such as wine and beer. Generally, for the analysis of saccharides, high-performance anion exchange chromatography (HPAEC) coupled with reflective index (RI) detection has been widely used (12,13). However, RI detection is not compatible with gradient elution and is sensitive to temperature. In particular, because the fermented products often contain a variety of ingredients, separation of the components in a sample using a method such as high-performance liquid chromatography (HPLC) with gradient elution is desirable for fine analysis of the saccharides in fermented foods and liquors. Carbohydrates in beer and wine have been detected with high resolution by an HPLC technique with gradient elution and evaporative light scattering detection (ELSD) (14,15). Hayakawa et al. (16) have also analysed sake for oligosaccharides using HPLC coupled with a polarized photometric detector

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TABLE 1. List of the maltooligosaccharides with DP 2–8 used as the standards in this study and their analytical properties in the HILIC-TOF/MS method.

	m/z	Retention time (min)	Regression equation[a]	R^2 [a]	RSD (%)[b]		LOD (ppm)[c]
					1 ppm	5 ppm	
DP 2	349.1322	12.0	y = 389243x + 845759	0.9967	2.64	5.15	0.10
DP 3	511.1850	12.6	y = 199824x − 74788	0.9995	0.89	1.72	0.03
DP 4	673.2378	13.4	y = 79976x − 169429	0.9963	6.95	2.09	0.25
DP 5	835.2906	14.3	y = 30690x − 64980	0.9952	3.39	1.75	0.12
DP 6	997.3435	15.2	y = 13723x − 25136	0.9964	7.92	3.65	0.29
DP 7	1159.3963	16.2	y = 7486x − 17809	0.9965	18.47	9.50	0.95
DP 8	1321.4491	17.0	y = 2785x − 14085	0.9981	N. D.[d]	8.12	1.02

[a] Regression equations and R^2 score of the regression curves were determined in the concentration range of 1–100 ppm except for DP 8 (5–100 ppm).
[b] RSDs(%) were calculated based on the repeated (n = 7) analysis.
[c] LODs were determined with following equation (28): LOD (n = 7) = 3.89 × standard deviation/slope of standard curve. For calculation, standard deviation of repeated analysis of 1 ppm samples (DP 2–7) and 5 ppm samples (DP 8) were used.
[d] N.D. means not detected.

(PPD) and detected oligosaccharides with DP values over 4. They suggested that the oligosaccharides profile in a sake mash at the initial and final stages are different; however, the exact DP values of the detected oligosaccharides were not determined.

Recently, pulsed amperometric detection (PAD) has become a popular and universal technique for oligosaccharide detection due to its compatibility with gradient elution and picomolar sensitivity (17,18). HPAEC method includes normal phase separation of oligosaccharides under very high pH (>12), which potentially induces epimerization or degradation of the sample oligosaccharides (19). Separation of oligosaccharides under reversed phase conditions is achieved by pre-column derivatization of oligosaccharides, which has been applied to the analysis of beer oligosaccharides (20,21).

Mass spectrometry (MS) is another method of choice for oligosaccharide detection for its potential in elucidating molecular species based on the *m/z* value of the detected ion without standard samples since oligosaccharide standards with a DP value over 8 are not readily available. Matrix-assisted laser desorption ionization (MALDI) with time-of-flight/mass spectrometry (TOF/MS) has been used for the oligosaccharides with a wide range of DP values in beer samples (21,22). A flow injection MS method has also been used for the oligosaccharide detection in beer (23). Moriwaki et al. (9) detected compounds in sake whose *m/z* were identical to the *m/z* of maltooligosaccharides with DP > 5 by using a flow injection MS method. Although these methods are powerful techniques for characterizing the oligosaccharide profile of a sample, both methods are generally unsuitable for quantification because the compounds in a sample are not separated, resulting in competitive ionization and consequent ion suppression.

In this study, we detected a series of oligosaccharides with DP up to 18 in sake by using a simple hydrophilic interaction liquid chromatography (HILIC)-TOF/MS method. We reveal the profile of the oligosaccharides in sake for the first time and suggest that the oligosaccharides have a branched structure.

MATERIALS AND METHODS

Sake samples The following commercially purchased sakes were used: sake A (*junmai* grade, rice polishing rate of 60%), sake B (*junmai* grade, rice polishing rate of 60%), sake C (*junmai* grade, rice polishing rate of 70%), sake D (*junmai daiginjo* grade, rice polishing rate of 50%), sake E (*daiginjo* grade, rice polishing rate of 35%), and sake F (nonpremium sake, rice polishing rate of 65%). Sake A was used as the representative sake in this study, and the others were used for comparison of the oligosaccharide profiles. *Junmai* grade sake, *junmai daiginjo* sake and *daiginjo* sake are premium grade sakes whose brewing quality requirements are defined by National Tax Agency Notice "Sake brewing quality labeling standards". Simply, the definitions are as follows; *junmai* grade sake is made only from rice, *koji* and water, *junmai daiginjo* grade sake is a kind of *junmai* sake made with rice whose polishing rate is no more than 50%, and *daiginjo* sake is made with rice whose polishing rate is no more than 50% and is allowed to contain additional brewers' alcohol in limited extend, respectively. Throughout this study, sake samples were stored at 4°C until used.

Sample preparation for HILIC-TOF/MS analysis All of the standard oligosaccharides were purchased from Wako Pure Chemical Industries (Osaka, Japan) except for maltotriose (SERVA Electrophoresis GmbH, Heidelberg, Germany) and maltooctaose (Carbosynth Limited, Berkshire, UK). The oligosaccharides, maltose (DP 2), maltotoriose (DP 3), maltotetraose (DP 4), maltopentaose (DP 5), maltohexaose (DP 6), maltoheptaose (DP 7), and maltooctaose (DP 8) were diluted in Milli-Q water to 1, 5, 10, 25, 50, and 100 ppm to prepare mixtures of the standard oligosaccharides (Table 1). To prepare the sake sample for HILIC-TOF/MS, 20 mL of commercially purchased sake were concentrated to less than 4 mL using a rotary evaporator. The sample was then diluted with Milli-Q water to a volume of 8 mL (a 2.5-fold concentrated sake sample). For the detection of the oligosaccharides by HILIC-TOF/MS, the 2.5-fold concentrated sake sample was diluted with Milli-Q water to a 32-fold diluent of the original sake in volume and then used for detection of the oligosaccharides with DP 2–11. For the detection of oligosaccharides with DP 12–18, the 2.5-fold concentrated sake sample was used. Comparison of the retention time of the oligosaccharides in sake and the standard oligosaccharides was carried out with an 80-fold dilution sake sample, 25 ppm of standard oligosaccharide solution, and a mixture of the sake sample and standard oligosaccharides. The mixture was prepared by mixing aliquots of the 40-fold diluted sake sample and the 50 ppm standard oligosaccharides. For the spike test and the estimation of the amount of oligosaccharides in the six sakes, 20-fold, 80-fold, and 500-fold diluted samples were prepared from the 2.5-fold concentrated sake samples and used for analysis of the oligosaccharides with DP 5–8, DP 3 and 4, and DP 2, respectively. All of the samples for HILIC-TOF/MS analysis were filtered through a Millex 0.45-μm pore size syringe filter (Merck KGaA, Darmstadt, Germany) before use.

HILIC separation For the separation of the oligosaccharides, HPLC was performed on an Agilent Technology 1260 Infinity (Agilent Technology, Santa Clara, CA, USA) equipped with a Shodex Asahipak NH2P-50 4E column (16) (4.6 mm × 250 mm, particle size 5 μm, Showa Denko, Tokyo, Japan). Milli-Q water containing 0.05 mM of lithium (24) chloride (purity >99.0%, Wako Pure Chemical Industries) was used as mobile phase A, and acetonitrile (Wako Pure Chemical Industries, LC/MS grade) was used as mobile phase B. The gradient elution parameters were optimized as follows: 0–5 min, 50% B; 5–20 min, 50–5% B; 20–25 min, 5% B; 25–26 min, 5–50% B; and 26–46 min, 50% B. The flow rate was 0.3 mL/min, the sample injection volume was 5 μL, and the column temperature was set to 40°C.

TOF/MS analysis Agilent Technology 6530 Accurate-Mass Q-TOF LC/MS (Agilent Technology) with electrospray ionization (ESI) was used with following settings: a positive ESI polarity, a drying gas temperature of 320°C, a flow rate of 8 L/min, a sheath gas temperature of 350°C, a sheathe gas flow of 11 L/min, a capillary voltage of 3500 V, and a nozzle voltage of 1000 V. The mass detection range was from *m/z* 150 to 3200. The introduction of LC eluate into the ESI was limited to 12–22 min to circumvent contamination by glucose and disaccharides when a concentrated sample was analysed. The LC/MS System was operated on Agilent Mass Hunter Workstation Data Acquisition with a data acquisition rate of 1 spectra/s, and data analysis was performed on Agilent Mass Hunter Qualitative Analysis B.06.00 (Agilent Technology). In the fragmentation analysis, ions with *m/z* values of 511.1850, 673.2379, 835.2907, 997.3435, 1159.3963, and 1321.4492 were targeted as precursor ions. The collision energy was set to 50 eV to generate fragment ions coincide with the fragment ions of oligosaccharides reported by Domon and Costello (25). The other parameters for the HILIC and TOF/MS system settings were the same as described above.

Partial purification of the oligosaccharides in sake Elimination of glucose from sake sample was conducted with the activated charcoal according to reference (26) with modification. The sake F (500 mL) was dealcoholized using a rotary evaporator and resulting dealcoholized sake was then diluted to 500 mL, and mixed with 100 g of activated charcoal (liquid chromatography grade, Wako Pure Chemical Industries). The mixture was then filtered through a paper filter (No. 2, Advantec, Tokyo, Japan) using a Buchner funnel and was rinsed with 1 L of Milli-Q water to remove glucose from the charcoal. The separated charcoal was mixed

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